Supplemental Data

The Dynein Light Chain Tctex-1

Has a Dynein-Independent Role

in Actin Remodeling during Neurite Outgrowth

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Supplemental Experimental Procedures

siRNAs, Plasmids, and Antibodies

Phosphorothioate (*)-modified AS oligos (rat Tctex-1: 5′-TGCTCACTTCATCCACA*A*C*; control oligo: 5′-TGTCACTTCTCACACC*A*A*) were purchased from BioSource (Camarillo, CA). Tctex-1 siRNA oligonucleotides (GUCAACCAGUGGACCACUAdTdT; UAGUGGUCCACUGGUUGACdTdT) were purchased from Qiagen-Xeragon (Germantown, MD). The Tctex-1-siRNA plasmid was constructed in pBS/U6 vector (gift of Dr. Y. Shi, Harvard) according to the procedures described in Xia et al. (2003), using 5′-GGTTACACACCGCAAGTTCCCCATGGGGAACTTGCGGTGTGTAACCCTTTTTA and 5′-AGCTTAAAAAGGGTTACACACCGCAAGTTCCCCATGGGGAACTTGCGGTGTGTAACC as targeting sequences. The scramble control-siRNA plasmid was purchased from Ambion Co. The DNA fragments containing U6-Tctex-1-si and U6-control-si were inserted into pCAGIG vector (kind gift of Dr. Connie Cepko) in which the GFP-cDNA is under the control of chick actin-minimal CMV (CAG) promoter; the resulting plasmids were referred to as Tctex-1-si/GFP and control-si/GFP, respectively. Flag-DIC expression vector was constructed by inserting PCR fragment (5′-GTTGAGCTGTAACCGGGGTCTG; 5′-CGGGATCCGCATGTCAGACAAAAGTGAATTAAAAG) of rat DIC2 into pRK5 vector. Autosequencing was used to confirm the inserted sequences. Expression vectors encoding GFP pEGFP-C1 (BD Biosciences, Palo Alto, CA), FLAG-Tctex-1 (Chuang et al., 2001), myc-p50 dynamintin (gift of Dr. Richard Vallee, Columbia University), YFP-GalT (Rosso et al., 2004), GFP-DIC (gift of Dr. T. Schorer, Johns Hopkins University [King et al., 2003]), DHC-siRNA (gift of Dr. L.H. Tsai, Harvard; [Shu, 2004]), myc-Rac1 L61 (gift of Dr. L. Luo; Stanford [Nakayama, 2000]), HA-Rho G14V, and HA-Rac1T17N (Gutheria) were used. mAbs recognizing Flag (clone M2), tyr-tubulin (clone TUB1A2), MAP2 (clone AP20), and DHC and rabbit anti-Flag Ab were from Sigma (St. Louis, MO). mAbs for myc (clone 9E10) and HA (clone 12CA5) were from Santa Cruz Biotechnology (Santa Cruz, CA). mAbs for DIC and TuJ1 were from Chemicon (Temecula, CA), Tau1 mAb was from Roche (Indianapolis, IN), p150glued mAb was from BD Biosciences, rat mAb YL1/2 against tyr-tubuline was from Dr. G. Gunderson (Columbia University), and rabbit anti-GFP Ab and Alexa dye-conjugated secondary Abs were purchased from Molecular Probe (Eugene, OR). Affinity-purified anti-Tctex-1 rabbit IgG was described in Tai et al. (1998).

Supplemental References

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Supplemental Table S1. Knockdown Effect of Endogenous Tctex-1 Level in AS- or siRNA-Transfected Neurons by Quantitative Fluorescence Measurements

	Control AS	**Tctex-1 AS**	**Control-si/GFP**	**Tctex-1-si/GFP**
Tctex-1	136 ± 24	12 ± 8*	112 ± 14	8+6*
βIII-tubulinTuJ-1	146 ± 8	138 ± 14	142 ± 6	141 ± 9
Tyr-Tubulin	224 ± 6	228 ± 12	234 ± 10	242 ± 12
GFP	ND	ND	240 ± 18	235 ± 17

Cultures were treated/transfected with indicated AS/plasmid 12-15 hr after plating; they were fixed 20 hours later and processed for immunofluorescence, and quantitative fluorescence measurements were performed using Metamorph software. All measurements were performed in stage 2 cells.

Each value represents the mean ± SEM. Values are expressed in pixels from 8 bit images (0, black; 255, white). Measurements were taken from the total cell body area.

*Values significantly different from those of the corresponding control group. A total of 50 cells were analyzed from each experimental condition.

Supplemental Table S2. Effect of Tctex-1 Suppression on Neuronal Shape Parameters

	Diameter $_{ave}$ (μm)	Length $_{ave}$ of Minor Neurites	Axonal Length $_{ave}$ (μm)	% Cells Stage 2 & 3
Treatment A				
Control AS	1.4 ± 0.4	25 ± 7.2	118 ± 8.4	50 ± 3 (2) 43 ± 3 (3)
Tctex-1 AS	0.38 ± 0.06*	6.9 ± 2.5*	ND	33 ± 2.1 (2)* 2 ± 0.2 (3)*
Control-si/GFP	1.2 ± 0.4	28 ± 8.4	124 ± 12.6	48 ± 4 (2) 41 ± 7 (3)
Tctex-1-si/GFP	0.34 ± 0.04*	5.8 ± 1.6*	ND	26 ± 8 (2)* 4 ± 0.8 (3)*
Treatment B				
Control AS	ND	42 ± 8.2	240 ± 22	12+2.0 (2) 85 ± 7.0 (3)
Tctex-1 AS	ND	26 ± 6.4	88 ± 14*	66 ± 8.0 (2)* 18 ± 4.0 (3)*
Control-si/GFP	ND	38 ± 6.6	218 ± 24	14 ± 4.0 (2) 82 ± 8.0 (3)*
Tctex-1-si/GFP	ND	27 ± 9.3	76 ± 18*	62 ± 8.0 (2)* 14 ± 7.4 (3)*

At 2 hr (Treatment A) or 15 hr (Treatment B) after plating, cultures were treated with control or Tctex-1 AS oligonucleotides or transfected with control-si/GFP or Tctex-1-si/GFP plasmid. Transfected cells were fixed 20 hr later and processed for immunofluorescence, and their morphometric parameters were analyzed by Metamorph software. Each value represents the mean ± SEM.

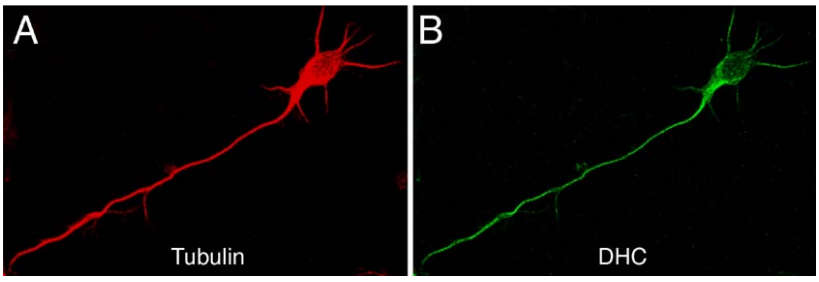
*Values significantly different from those of the control group ($p < 0.05$). 50-75 cells were analyzed for each experimental condition.

Supplemental Table S3. Effect of Tctex-1 Overexpression on Neuronal Shape Parameters

Groups	Total No. of Neurites	% Neurons w/only Minor Neurites	Length $_{ave}$ of Minor Neurites	% Neurons w/Axon-like Neurites	Length $_{ave}$ of Axon-like Neurites	No. of Axon-like Neurites
GFP+Tctex-1						
12 hr	5.4 ± 0.8*	75 ± 7*	25 ± 7	18 ± 2*	80 ± 8	2.2 ± 0.2*
18 hr	6.2 ± 1.2	12 ± 4*	34 ± 2	65 ± 12*	97 ± 9	3.8 ± 0.6*
24 hr	6.4 ± 0.8	2 ± 0.4*	35+7	92 ± 6	185 ± 15*	4.4 ± 0.8*
30 hr	6.6 ± 0.8	0.8 ± 0.4*	36 ± 4	98 ± 0.8*	234 ± 28*	5.2 ± 0.6*
GFP+DIC						
12 hr	3.8 ± 0.2	52 ± 6	14 ± 4	2.8 ± 0.2	60 ± 12	1.0 ± 0.0
18 hr	4.6 ± 0.2	60 ± 4	22 ± 8	14.5 ± 7	82 ± 8	1.1 ± 0.5
24 hr	5.2 ± 0.4	59 ± 7	32 ± 5	41 ± 6	122 ± 16	1.2 ± 0.6
30 hr	6.4 ± 0.8	18 ± 6	38 ± 12	82 ± 8	156 ± 14	1.2+0.4

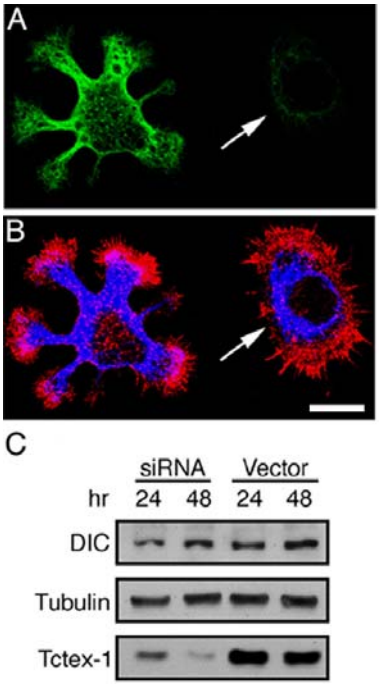
Cultures were cotransfected with GFP plus Flag-Tctex-1 or Flag-DIC (2 µg each) 2 hr after plating. Transfected cells were fixed at 12, 18, 24, and 30 hr time points, processed for immunofluorescence, and measured for morphometric parameters. Cells transfected with GFP alone (4 µg DNA) showed morphological parameters identical to those of neurons coexpressing GFP and Flag-DIC (data not shown). An axon was considered any neurite having a length of at least 50 µm and displaying Tau1 immunofluorescence. Each value represents the mean ± SEM.

*Values significantly different (at least $p < 0.05$) from those of the control group. 50-75 cells were analyzed for each experimental condition.



Supplemental Figure S1. DHC Distribution in Hippocampal Neuron Cultures

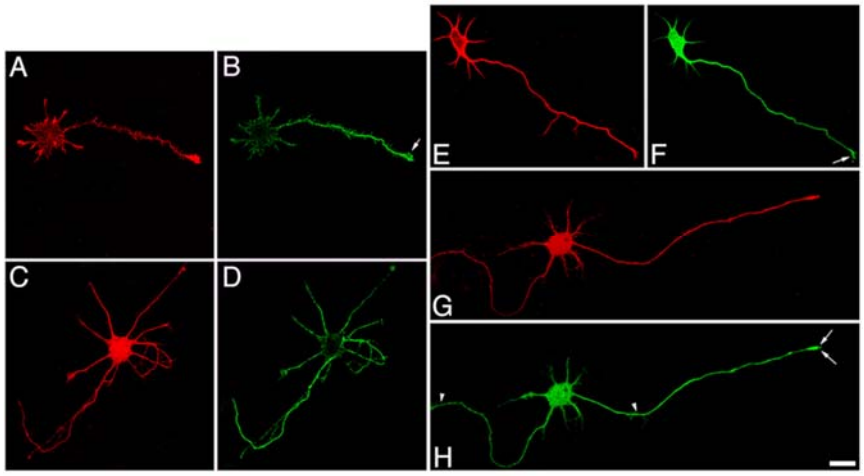
Confocal images of a stage 3 neuron colabeled for tubulin (A) and DHC (B). DHC labeling is homogenous throughout all neurites without a particular enrichment at the distal axonal shaft and growth cone.



Supplemental Figure S2. Tctex-1-siRNA Significantly Reduced Endogenous Tctex-1 Level

(A and B) Neurons were transfected with Tctex-1-siRNA or control oligonucleotide 2 hr after plating. The cells were fixed and immunostained 24 hr after transfection. The confocal image shows that the Tctex-1-siRNA transfected cell (arrow) displayed less endogenous Tctex-1 labeling (A, green) and lacked typical neurites (B, red for actin; blue for tubulin). This was in contrast to a neighboring nontransfected cell exhibiting several minor neurites with similar lengths, typical of stage 2. Scale bar equals 10 μm.

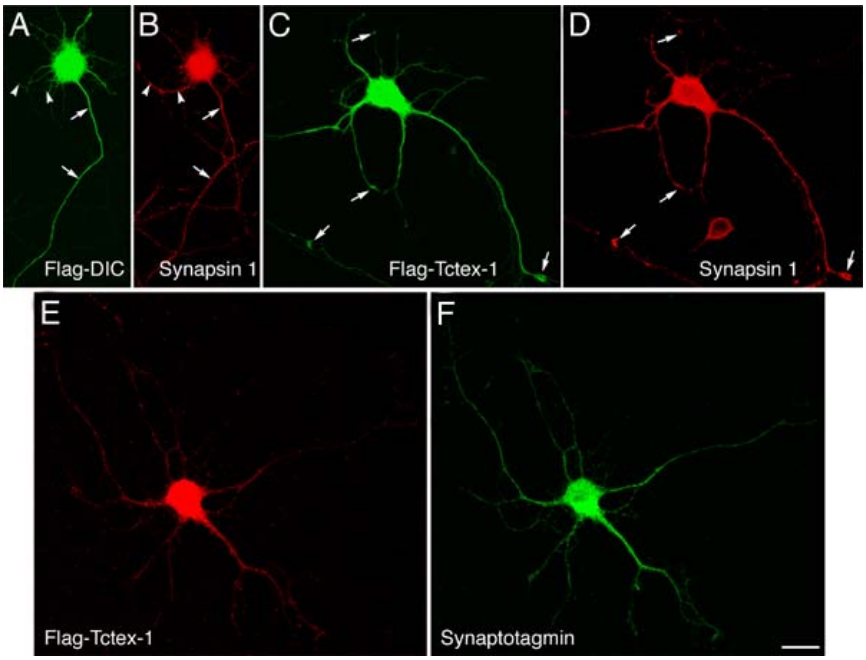
(C) Immunoblotting analyses demonstrated that the Tctex-1 siRNA-plasmid, but not the empty vector pBSU/6, specifically reduced the expression level of cotransfected rat Tctex-1 in human embryonic kidney 293T cells without affecting the levels of endogenous dynein DIC or tubulin.



Supplemental Figure S3. Tctex-1-Transfected Neurons Exhibited Multiple Neurites Expressing Axonal Markers APC and Cdc42

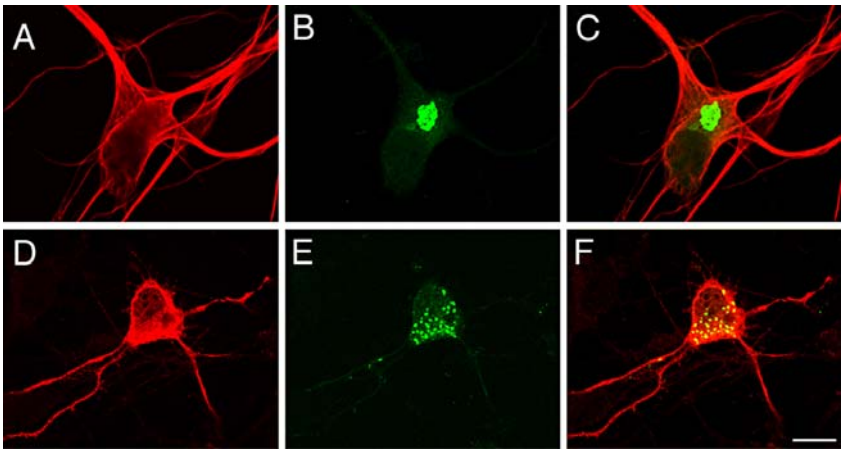
Neurons were either untransfected (A, B, E, F) or transfected with Flag-Tctex-1 cDNA (C, D, G, H) 2-4 hr after plating and fixed 18-20 hr later. They were labeled for actin (red in [A]), tyr-tubulin (red [E]), and Flag (red in [C] and [G]) together with Cdc42 (green in [B] and [D]) or APC (green in [F] and [H]). Note

that in nontransfected cells, Cdc42 and APC were predominantly localized to the single axon and its growth cone (arrows in [B] and [F]). By contrast, Tctex-1 transfected neuron exhibited several long axon-like neurites that intensely stained for Cdc42 and APC. Scale bar equals 10 μm.



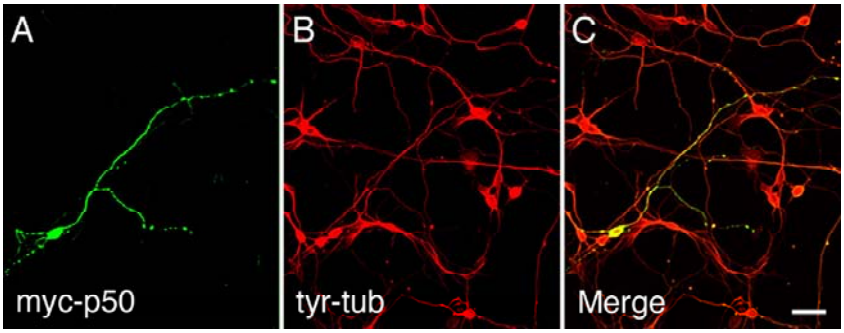
Supplemental Figure S4. Overexpression of Tctex-1, but Not DIC, Induced Multiple Neurites Expressing Axonal Markers Synapsin 1 and Synaptotagmin

Dissociated neurons were transfected with Flag-DIC (A, B) or Flag-Tctex-1 (C-F) 4 hr after plating. Cells fixed 22-24 hr posttransfection were colabeled for Flag (A, C, E) and synapsin 1 (B, D) or synaptotagmin (F). Note that in the Flag-DIC-transfected neuron, synapsin 1 is primarily localized to the axonal shaft (arrow); minor neurites only display faint staining. The arrowheads in (A) and (B) point to the axonal processes extended from the neighboring nontransfected neurons. By contrast, all of the processes extended by Tctex-1-transfected neurons display a high synapsin 1 immunofluorescence that extends up to their tips (arrows). Neurons transfected with Tctex-1-Flag also display multiple synaptotagmin-labeled processes. Scale bar equals 10 μm.



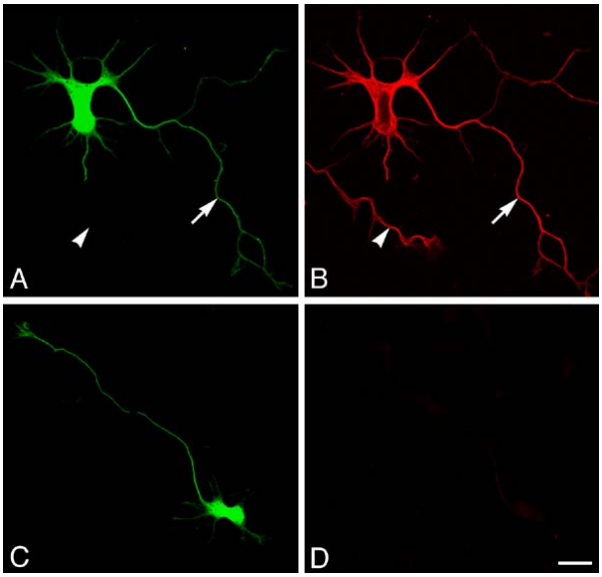
Supplemental Figure S5. p50 Overexpression Caused Golgi Fragmentation

3 DIV neurons were either transfected with YFP-GalT2 (A-C) or YFP-GalT2 together with myc-p50 (D-F). Cells fixed 18 hr later were immunolabeled for tyr-tubulin (red) and YFP (green) was directly visualized. Golgi apparatus was juxtanuclearly localized in YFP-GalT2 singly transfected cell (B), but became fragmented in myc-p50 transfected cells. Scale bar equals 10 μm.



Supplemental Figure S6. Neurons Overexpressed p50 Had Grossly Normal Neurite Development

Immunolabeling of myc (A) and tyr-tubulin (B) of 1 DIV neurons transfected with myc-p50. Scale bar equals 30 μm



Supplemental Figure S7. Silencing Effect of DHC-siRNA in Transfected Neurons

Confocal images of 1 DIV neurons transfected with GFP (A and B) or GFP together with DHC-siRNA (C and D) 2 hr after plating labeled for DHC Ab. In (A) and (B), a nontransfected cell (arrowheads) and a GFP-transfected cell (arrows) exhibit similar level of DHC immunofluroescence. By contrast, in (C) and (D), a DHC-siRNA/GFP-transfected neuron had almost undetectable level of DHC, even though this cell extends a single axon with multiple minor neurite. Scale bar equals 10 μm.